FINAL REPORT OF CONTRACT #3656 (06)

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2. Date of Contract:

Started - September 1, 1962
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3. Research Summary:

a. Animals
b. The Infectious Process
c. Protozoa
d. Plants
e. Insects

4. Graduate Students Trained Under the Contract:

a. Sadao Kotaka, Ph.D. 1965; from Tokyo University of Education, Tokyo, Japan.

b. Paul C. Andriese, Ph.D. 1967; from the University of California, Berkeley.

c. Present Locations: both men are currently associated with me at the Air Ion Laboratory, School of Public Health at the address given above.

5. Inventions:

a. Specific Ion Generators. Two devices were designed to generate particular ion species in a selected gas. The first model was designed to operate with a gas flowrate of approximately 60 cm^3 min^{-1}. Its experimental application was described by Krueger, et al, (1964). However, the need was apparent for a specific ion generator with a vastly reduced gas

flowrate. Such a device was designed, fabricated and tested for gas flowrates as low as 5 cm^3 min^{-1} and appears to have wide experimental applicability. Ion pairs are formed in a pure gas within a tritium-lined duct where ion separation is effected by the applied bias voltage. Unipolar ions are projected from the generator by the bias voltage and the moving air stream. A peripheral air supply acts as a carrier stream in addition to diluting the selected gas to normal levels. The instrument is capable of high unipolar ion production or the production of experimental atmospheres where both types of gaseous ions are present. It is hoped that this generator will help answer some of the questions being raised in connection with the chemical nature of air ion action. (See A.P. Krueger, S. Kotaka and P.C. Andriese 1966, "Studies on the Biological Effects of Gaseous Ions, A Review," Vol. 1, Special Monograph Series, Biometeorological Research Center, Leiden).

b. Plexiglass Exposure Apparatus: Cylindrical Housing. This equipment was designed to house small animals in experimental atmospheres modified only by the addition of selected gaseous ions to the slowly-moving and unobstructed air stream. The animals are supported at the bottom of a smooth-sided cylindrical chamber. Normal animal wastes drop through the open lattice of the floor to the waste collector and dryer below. Thus, artificially generated gaseous ions are added to clean, unpolluted air and are moved through the environment of the experimental animals without significant ion loss to the surfaces of the holding unit itself.

c. Disposition of Inventions. These have been included in our publications in compliance with the recommendation of Dr. Hundley, Acting Surgeon General, USPHS, in a letter to Mr. Owens of the University of California Patent Office on December 9, 1965 with copies to Mr. Klein of ONR.

3. Research Summary.

a. Study of the Effects of Gaseous Ions on Higher Animals.

Early experiments conducted prior to the contract period showed that unipolar positively ionized air produced the following changes in the tracheas of living rabbits, mice, rats, guinea pigs and monkeys: 1) decreased ciliary activity, 2) contracture of the posterior tracheal wall, 3) exagerated vulnerability to trauma and 4) vasoconstriction. The first three effects were seen in excised sections of trachea. Negative ions reversed these effects. It was found that CO_2^+ ions are responsible for the physiologically adverse effects of positively ionized air, while O_2^- ions are the biologically active species in negatively ionized air. Further, effects of CO_2^+ ions on the trachea appeared to imitate the effects produced by the intravenous injection of 5-hydroxytryptamine (5-HT or serotonin). These effects could be reversed by treatment with O_2^- ions. From these observations the 5-HT hypothesis developed: that CO_2^+ ions bring about a local accumulation of 5-HT in the trachea, that 5-HT is the major immediate cause of the functional changes produced by positive ions, and that the action of O_2^- ions in reversing positive ion effects depends upon accelerating the rate at which free 5-HT is oxidized. The hypothesis was tested by the use of the two drugs, reserpine and iproniazid. Reserpine causes tissue 5-HT depletion, an effect similar to that produced by negative O_2^- ions, while iproniazid, the monamine oxidase inhibitor, induces the accumulation of 5-HT in the tissues, an effect similar to that produced by CO_2^+ ions. Assay of 5-HT from the blood of mice further supported the earlier findings by showing that the blood concentrations of 5-HT were elevated when mice were exposed to CO_2^+ ions and were lowered by O_2^- ions. It has also been demonstrated that the elevation of the blood levels of 5-HT which occurs in the mice during exposure to CO_2^+ ions does not take place when non-ionized CO_2 or CO_2^- ions are added to the experimental atmosphere at comparable levels. Additional support for the 5-HT hypothesis has come from investigators in other countries and is discussed in our published work.

b. Study of the Effects of Gaseous Ions on the Infectious Process.

Very little systematic work has been done on the effects of small ions on infectious processes in animals. For the past two years Krueger and Levine have studied the effect of small ions on the course of coccidioidomycosis produced in mice by intranasal administration of measured numbers of arthrospores of Coccidioides immitis. Continuous exposure of the animals to small positive air ions resulted in changes not ordinarily observed in murine coccidioidomycosis: 1) within the first seven days after infection some of the mice developed marked listlessness, ruffled coat, weight loss and evident malaise. These animals died between the 12th and the 17th day. Significant numbers of control animals did not die until the 18th day. 2) By the 30th day a late increase in deaths among ion-treated mice brought the cumulative mortality rate to 55% in contrast with the rate of 30% in the controls. This difference was significant at the 97.5% level. 3) In a similar experiment with arthrospores possessing an abnormally high pathogenicity, early deaths among ion-treated mice again occurred. 4) Exposure of infected mice to an electrical field identical with that used in ion treatment did notinfluence the mortality rate. 5) Fungal counts performed at intervals of homogenates of lungs, livers, and spleens removed from control and ion-treated infected animals gave no evidence that treatment with positive air ions depressed host defense in the direction of facilitating fungal proliferation in the lungs as well as fungal spread to extrathoracic sites.

c. Studies of the Effects of Air Ions on Protozoa.

The free-living amoeba Hartmannella rhysodes was selected for study because it may be cultured both on an agar surface and in liquid media. Some migration characteristics were defined and the effects of ion exposure were expressed in terms of these characteristics. Equipment was designed to facilitate routine observation of H. rhysodes on a modified agar plate (black) and the recording of tracks formed during the ion exposure periods. An open plate exposure apparatus and its supporting system were also designed, fabricated and tested. It includes 1) plate temperature control by the control of the environmental humidity and temperature, and draft-free circulation, 2) A ground circuit from the plate to earth provides constant data on ion flow, and 3) the control of stray electrical fields. Using the rate of migration of the organisms over the agar surface as an indicator of physiological changes, it was found in these early studies that amoebae in the presence of a positive ion stream migrated more slowly than the controls whose environment was identical to that of the test group except that no ions were added to the environment. Though some study sets showed as much as a 29% reduction in locomotion, the reduced rate in other sets were not statistically significant. Collectively, however, the data indicated a mean reduction of 5.2% which is significant at the .05 level.

d. Studies of the Effects of Air Ions on Higher Plants.

Since September 1960 we have studied the effect of gaseous ions on representative higher plants including oat (Avena sativa), barley (Hordeum vulgaris), lettuce (Latuca sativa) and pea (Pisum sativum). Seedlings were raised in either washed sand infused with a chemical nutrient solution or 15 liter tanks containing a similar culture medium. A series of experiments was performed in cubicles (125 ft^3 capacity) supplied with air purified by passing it through an electrostatic precipitator, an activated carbon filter and an air conditioner. Conditions of lighting, temperature and humidity were maintained at a constant level.

One cubicle was supplied with negatively ionized air, the second with positively charged air and the third cubicle served as a control environment containing an average of 250 positive ions and 200 negative ions per cc of air.

Experiments were also performed in a greenhouse supplied with purified air and the seedlings were grown in inverted clear plastic canopies with ion generators mounted in the top. The minimum temperature in the greenhouse was maintained at 25°C but maximum temperature varied with the season.

It was found that air ions of either charge induce statistically significant increases in growth rate as determined by seedling length, integral elongation, fresh weight and dry weight. The growth increase approximately parallels the ionic density and is naturally accompanied by a proportional increase in the flow of electric current to ground. Analytical data of ion-treated plants showed that there is no deviation in total nitrogen, protein, total sugars, and reducing sugars from the compositions of untreated control plants.

In order to find the biochemical mechanisms which are responsible for the growth stimulation by air ions a series of experiments has been conducted. It was observed that development of iron chlorosis in barley seedlings cultivated in an Fe-free culture medium is markedly accelerated by exposure of the plants to air ions of either charge. Chlorosis is a condition in which the foliage of the plant becomes yellow or colorless because of interference with chlorophyll formation. As the concentration of chlorophyll drops with the onset of chlorosis, the concentration of cytochrome c rises markedly. When seedlings are grown in an iron-containing medium and are exposed to positive or negative ions during growth, the typical ion-induced acceleration in growth rate is accompanied by stimulation of cytochrome c synthesis but no chlorosis develops and there is no essential difference in the concentration of chlorophyll among exposed and control plants.

From these data we surmised that gaseous ions might affect iron metabolism in plants. In order to explore this possibility a series of experiments was performed to test the effects of air ions on the "active" and "residual" iron of barley seedlings during the course of iron chlorosis. Active iron is the fraction concerned with the production of chlorophyll; it occurs in the chloroplasts and is soluable in 1 N Hcl. Residual iron is present outside the chloroplasts; it is not related to chlorophyll production nor is it soluble in 1 N Hcl. It was found that negative or positive ions significantly decrease the active iron content of the seedlings during the period when the chlorophyll content decreases, whereas there occurs an increase in residual iron and in the cytochrome fractions of the seedlings. We also obtained some evidence that the increase in residual iron content involves not only cytochromes bu other iron-containing enzymes as well.

Based on these data we have proposed the hypothesis that a part of the iron originally contained in the seed exists in a non-specific form and can be used as required for any purpose (free-state iron). When seeds are soaked in water, a fraction of the iron is diverted to certain enzyme system(s) concerned with germination. The remainder is used in a balanced fashion for cytochrome synthesis and for synthetic processes leading to chlorophyll formation. If the seeds are grown in an iron-free environment and are exposed to high concentrations of air ions of either charge, the balance normally prevailing between the two processes is upset, endogenous iron is diverted preferentially to the production of cytochromes and other iron-containing enzymes with the result that insufficient iron is available for synthesis of chlorophyll; consequently chlorosis develops. Under the condition usually prevailing in nature external iron sources are available, the normal chlorophyll formation proceeds and no chlorosis occurs. The synthesis of cytochromes and other iron-containing enzymes is stimulated by air ions and the plant is sufficiently supplied with metabolic equipment essential for the support of an increased rate of growth. It can be assumed that the site of air ion effects may be the regulatory systems which control

iron metabolism in the seed and young seedling.

Further, two additional observations were made that are compatible with our hypothesis. Isotopic experiments using Fe^{55} showed that the treatment of very young seedlings with negative or positive air ions increases the rate of uptake of exogenous iron. It was also found that air ions of either charge increase the oxygen consumption of barley seedlings. Based on these data we can assume that an accelerated iron uptake provides for the increased production of the metabolically important factors, that is, iron-containing compounds which comprise the residu' iron fraction and this leads as expected to accelerated oxygen consumption serving the increase in the basic metabolic rate in plants. Thus the basic biochemical equipment is provided to support an increase in growth rate.

Since the chemical structure of indole-3-acetic acid (IAA) is similar to that of 5-hydroxytryptamine (5-HT) and since the latter compound is involved in the production of air ion effects on animals, it is reasonable to consider the possibility that IAA is concerned in air ion induced growth acceleration of higher plants. We developed improved analytical methods for IAA estimation in direct tests of air ion effects on the growth of Hordeum vulgaris. Although the air-ion-treated plants had a dry weight twice that of the controls, it was found that the active free- and inactive bound-IAA content of the plant tissues were not significantly influenced by exposure to ionized air.

Since the changes in plant physiology previously described are induced by exposing plants to negatively or positively ionized whole air, it was necessary to determine which ionic types are responsible for the effects. Using specific ion generators designed for this purpose, various gaseous ions were added to the atmosphere of plastic chambers in which barley seedlings were grown in sand culture containing a chemically defined nutrient solution. Moderate densities of O_2^- or O_2^+ ions in air containing an added 8% O_2 produced a marked increase in growth rate while a comparable number of CO_2^- and CO_2^+ ions in air containing 8% CO_2 inhibited growth. The growth inhibition by CO_2^+ was observed either under laboratory or greenhouse conditions.

We have observed that isolated chloroplasts suspended in a buffered sucrose solution shrank when stored for 48 hours at 4°C and swelled again when they were illuminated. The rate of swelling, determined microscopically or by a light-scattering technique was increased by the addition of ATP. Swelling of 80-90% of the chloroplasts occurred during illumination for 2 hours with 5,000 fc at 25°C. Inhibitor and other experiments showed that the effect involves the expenditure of energy from ATP hydrolysis. Incubation of the chloroplasts at 4°C in the dark with unipolar ionized air of either charge produced a measurable increase in shrinking-swelling action, leading to the conclusion that positive and negative gaseous ions present in the ionized air stimulate ATP metabolism. Production of ATP asc parallels the increased rate of shrinking-swelling brought about by exposure to air ions. We are currently performing a series of experiments in order to obtain more direct evidences of ATP involvement in the swelling-shrinking phenomenon.

e. Studies of the Effects of Air Ions on Insects.

The experimental data cited above indicate that cytochromes and related iron-containing enzymes are important in mediating air ion effects on higher plants. This consideration led us to find some other form of life known to be dependent on cytochromes for significant changes in its life cycle and at the same time suitable for testing the effects of exposure to atmospheric ions. We found this in the silk worm Bombyx mori L., whose larval growth and molting hormone is produced in the prothoracic glands or in other homologous thoracic organs. The primary function of this hormone is to stimulate the biosynthesis of cytochrome c and possibly other cytochrome components. Accordingly, during 1965 we conducted a series of experiments at Ibaraki National University in Japan to determine the effects of air ions of the growth of silkworm larvae and on their content of various iron - containing enzymes. Starting with uniform populations of silkworm eggs and exposing them to unipolar ionized air at 24°C and at 26°C, the following physiological changes were observed with either negative or positive air ion treatments: 1) a measurable acceleration in the hatching of eggs, 2) a marked increase in the formation of catalase, peroxidase and cytochrome c oxidase, 3) acceleration of the onset of spinning and 4) an increase in cocoon weight.

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